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Glucocorticoids Decrease Interleukin-6 Levels and Induce Mineralization of Cultured Osteogenic Cells from Children with Fibrous Dysplasia*

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ABSTRACT

Fibrous dysplasia (FD) is a progressive bone disease in which abnormal fibroblast proliferation results in the replacement of normal cancellous bone with an immature fibrous tissue that is poorly mineralized. The disease manifests itself in the monostotic form in which only one bone is involved and the polyostotic form in which multiple bones at different sites are affected. The McCune–Albright syndrome is a variation of the polyostotic form in which patients demonstrate a greater extent of bone involvement and a variety of endocrinopathies. Somatic activating mutations in the *GNAS* gene have been demonstrated in the fibrotic lesions of patients affected with either monostotic or polyostotic FD. The increased cAMP levels caused by the G-protein mutations lead to increased interleukin-6 (IL-6) levels in the affected tissues, resulting in abnormal osteoblast differentiation and increased osteoclastic activity. Utilizing cell culture techniques that have been developed for mammalian bone marrow stromal cells, we have successfully cultured osteogenic stem cells from the affected stroma of 11 FD patients. Cells cultured from patients with polyostotic FD showed a high frequency of the $G_s\alpha$ mutation, whereas cells from monostotic FD patients showed a low frequency of the mutation. Both the normal and FD cells displayed the osteogenic phenotype when exposed to medium containing glucocorticoids. Glucocorticoids also caused a dramatic inhibition of IL-6 mRNA and protein levels in osteogenic cells cultured from the FD patients. These findings suggest that chemical alteration of cellular function may lead to new treatment options for patients with FD. (J Bone Miner Res 1999;14:1104–1114)

INTRODUCTION

FIBROUS DYSPLASIA (FD) is a rare disease affecting the bones of children in which the normal bone marrow and cancellous structures are replaced with a dense stroma of fibrotic tissue and immature woven bone (reviewed elsewhere[1]). Within the fibrotic tissue are irregular and disordered spicules of trabecular bone, which are not interconnected. The stromal tissue is proliferative and expands at the expense of the remaining adjacent normal bone. The proliferative cells in the marrow stroma have been shown to express alkaline phosphatase (ALP), an early marker of osteoprogenitor cells.[2,3] Furthermore, Riminucci et al.[2] have demonstrated that the fibrotic lesions within the affected bones consist of mature, but abnormally differentiated, osteoblasts. Thus, FD of bone appears to be a disease of cells in the osteogenic lineage.[2] Affected bones are weak, and with the stress of weight bearing, or as a result of repeated fractures, are likely to develop significant deformities.

FD manifests itself in three forms. In the monostotic form, only one bone is affected, whereas, in the polyostotic

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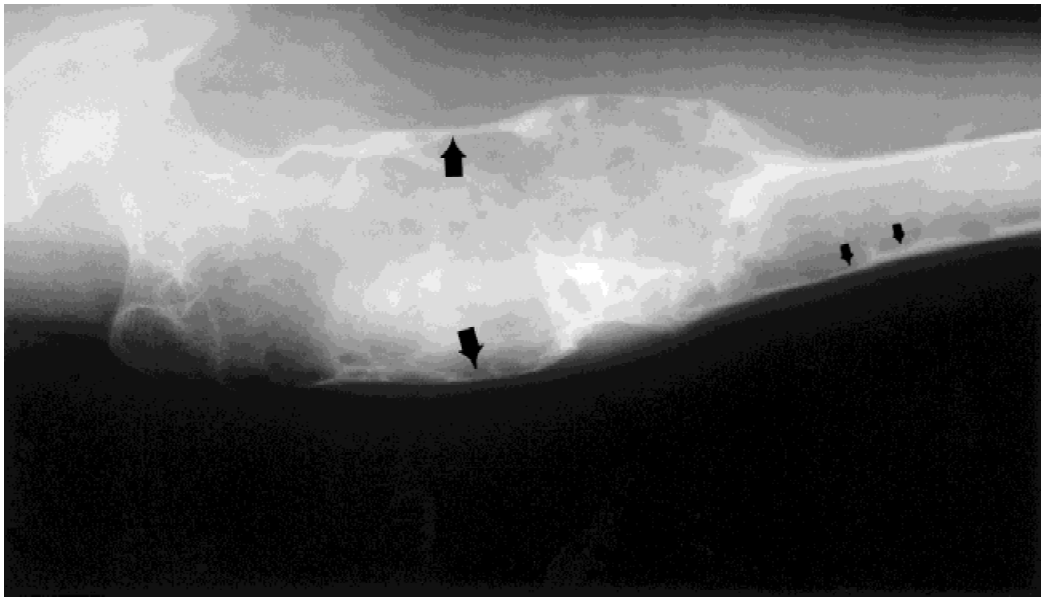

FIG. 1. Extensive enlargement and cortical destruction of the proximal right femur in Case 1 as a result of endosteal proliferation of FD. Arrows indicate areas of endosteal "scalloping" typical of this process.

form, multiple bones are involved. The McCune–Albright syndrome is a variation of the polyostotic form in which patients demonstrate a greater extent of bone involvement and a variety of endocrinopathies (reviewed elsewhere[4]). There is no evidence for genetic inheritance of this condition; rather, somatic mutations in the alpha stimulatory subunit of the G protein ($G_s\alpha$) have been demonstrated in varying amounts in affected tissues of McCune–Albright patients.[5–9] Similar mutations have been shown to occur in monostotic FD.[10] Either of two missense mutations, which result in substitution of the arginine 201 (Arg201) residue with either cysteine (Cys201) or histidine (His201), have been demonstrated in FD tissue. These amino acid substitutions result in inhibition of the GTPase activity of the G protein, which then causes prolonged stimulation of adenylate cyclase in the absence of hormonal exposure.[11]

Historically, treatment for FD has consisted of surgical removal of the abnormal tissue, placement of bone grafts, and use of internal fixation devices in an attempt to delay the onset of recurrent deformity. The natural history of the condition is one of recurrence, with resorption of grafts and repeated corrective surgeries for the patient. Recently, bisphosphonates have been used in treatment of FD, but on a very limited basis in children.[12–14] Furthermore, Meunier and coworkers[12,14] suggest caution in the use of bisphosphonates in patients with open physes, because the authors noted radiographic changes of uncertain long-term significance. We postulated that treatment of FD in children would be more effective if the preosteogenic cells could be induced to produce bone under the influence of chemical treatments. To screen potential therapeutic agents, we first developed a tissue culture model of FD by growing cells from the affected stroma of children with FD. It has been well established that there are osteogenic stem cells in the bone marrow of mammals, which appear to be responsible for bone remodeling and regeneration following fracture repair.[15–18] These fibroblastic cells retain the ability to form bone and cartilage in vivo but do not undergo osteogenic differentiation in vitro unless the culture medium is supplemented with dexamethasone.[15–19] We demonstrate that it is possible to culture this preosteogenic stem cell from the affected stroma of FD patients. Furthermore, we use a nonradioactive polymerase chain reaction (PCR)-based assay to show the presence of $G_s\alpha$ mutations in the cultured stromal cells from FD patients.

Interleukin-6 (IL-6) is a cytokine that is known to inhibit bone formation and stimulate osteoclast activity.[20–23] Increased osteoclastic activity has been observed in FD[12] and is attributed to the increased IL-6 levels found in the fibrotic lesions of McCune–Albright syndrome patients.[24] Recently, Haynesworth et al.[25] have shown that dexamethasone decreases expression of IL-6, IL-11, and leukemia inhibitory factor in normal human bone marrow stromal cells. We hypothesized that glucocorticoid treatment would decrease IL-6 production and induce mineralization in cultured stromal cells from FD patients.

MATERIALS AND METHODS

Cell culture

Eleven patients diagnosed as having FD by clinical, radiographic (Fig. 1), and histologic criteria were studied (Table 1). Each patient received a subjective clinical rating based upon clinical factors, including the age of the patient at first fracture, the frequency of subsequent fractures, the rapidity of bone deformation, and the rate at which cortical allografts were resorbed following reconstructive surgery. A rating of 10 was reserved for patients with the most severely aggressive clinical expression of the disease. FD tis-

TABLE 1. CLINICAL CHARACTERISTICS AND MUTATIONS OF THE FD PATIENTS USED IN THIS STUDY

Case number	*Gender*	*Age (years) at surgery*	*Severity**	*Diagnosis*†	*Mutation*‡
1	M	19.1	9	polyostotic FD	His201, 86%
2	F	6.3	8	McCune–Albright syndrome	Cys201, 81%
3	M	7.0	4	monostotic FD	His201, <1%
4	M	7.5	3	monostotic FD	His201, <1%
5	M	7.1	9	McCune–Albright syndrome	His201, 70%
6	F	11.1	6/7	McCune–Albright syndrome	Cys201, 53%
7	M	13.6	2	monostotic FD	Cys201, 17%
8	F	11.8	8	McCune–Albright syndrome	His201, 63%
9	M	8.5	8	McCune–Albright syndrome	ND
10	F	15.3	4	polyostotic FD	Cys201, 39%
11	M	14.2	9.5	McCune–Albright syndrome	Cys201, 42%§

* Severity was rated on a scale of 1–10, with 10 being reserved for the most clinically aggressive disease as outlined in the Materials and Methods.

† Diagnosis was made based on whether one (monostotic) or more (polyostotic) bones were involved and whether there was also endocrine involvement (McCune–Albright syndrome). All removed tissue was confirmed as FD by histological examination. Samples from Cases 3 and 4 with monostotic FD also showed cartilaginous components.

‡ The His201 and Cys201 mutations were detected after digestion of the region encompassing the $G_s\alpha$ mutation with *Nla*III and *Pvu*II, respectively. The percentage listed after the mutation is an estimate of the proportion of cultured cells that carry the $G_s\alpha$ mutation and was determined as described in the Materials and Methods.

§ This mutation was also detected in peripheral blood lymphocytes from this patient.

ND, not detected.

sue removed at the time of reconstructive orthopedic procedures was used for the isolation of stromal cells for subsequent culture. Bone marrow stromal cells were cultured from normal volunteers undergoing corrective orthopedic surgical procedures. No normal patients had evidence of metabolic bone disease, and patients were excluded if they were taking any medications. Informed consent was obtained for each individual in this study, which was approved by the Institutional Review Board of the Alfred I. duPont Hospital for Children.

Biopsied material commonly consisted of bone spicules and marrow cavity fluid. In two monostotic FD patients, cartilaginous components were present in the biopsied material. The bony spicules and cartilaginous material were minced, and cells were obtained from explant cultures of these tissue pieces. The liquid material was pipetted to break up cell aggregates, and 5 ml of this suspension was inoculated into a 75-cm^2 flask that contained 15 ml of alpha modified essential medium (α-MEM) supplemented with 15% fetal bovine serum (FBS) (Biocell, Rancho Dominquez, CA, U.S.A.), 50 mg/ml streptomycin, and 50 U/ml penicillin (GIBCO, Grand Island, NY, U.S.A.). Explant cultures were maintained in the same medium in 75-cm^2 flasks. All cultures were maintained at 37°C in a 95% air and 5% CO_2 atmosphere. The next day, the attached cells were washed twice with Hank's balanced salt solution to remove most of the nonadherent hematopoietic cells, and then fresh medium was added. The medium was changed every 3 days until the cells became confluent. The cells were subcultured once (passage 1) and then used for isolation of DNA as described below. Independent experiments revealed no differences in the properties of cells obtained from bone spicules, cartilage, or liquid material in terms of cell proliferation rates, frequency of the $G_s\alpha$ mutation, response to glucocorticoids, and degree of mineralization.

To determine doubling times of the various FD cell lines in relation to control cells, 5×10^4 cells from early passages[2,3] were inoculated into each of ten 25 cm^2-flasks. Cell numbers from two duplicate 25-cm^2 flasks were determined every 24 h for 5 days by use of a hemacytometer. Cell number versus hours in culture was plotted on a semilog scale, and doubling time was determined from the exponential phase of the growth curve.

For the mineralization assays, cells were plated from frozen stocks at 5×10^3 cells/cm^2 in 35-mm tissue culture dishes in α-MEM supplemented with 15% FBS, penicillin-streptomycin, 50 μg/ml L-ascorbate, and 10 mM β-glycerophosphate. Either 10 nM dexamethasone or 100 nM methylprednisolone was added to the medium from day 1 forward, and the medium was then changed three times per week. Mineralization of cultured cells was assessed by either staining cells for 3 minutes in 2% (w/v) alizarin red or by vonKossa's method for calcium detection.[26]

DNA isolation

Cells from a confluent 75-cm^2 flask (~2.5×10^6 cells) were removed from the substratum by trypsination and collected by centrifugation at 1000 rpm. The cell pellet was then resuspended in 600 μl of cell lysis solution (Gentra Systems, Inc., Minneapolis, MN, U.S.A.) and processed according to the manufacturer's instructions. DNA was prepared from FD tissue by using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) to homogenize 100 mg of tissue in 9 ml of RBC lysis solution (Gentra Systems, Inc.), and the sample was then processed according to the manufacturer's suggestions. DNA concentration and purity were assessed by determining absorbance at 260 nm and 280 nm.

TABLE 2. PRIMER SEQUENCES FOR PCR AMPLIFICATION OF THE REGION CONTAINING THE $G_s\alpha$ MUTATIONS AND FOR RT/PCR ANALYSIS

Primer	*Gene*	*GenBank accession number, position*	*Sequence**
HGNASF1	GNAS	M21142, 377–398	5′ACTGTTTCGGTTGGCTTTGGTG3′
HGNASR1	GNAS	M21142, 544–565	5′AGGGACTGGGGTGAATGTCAAG3′
HGNASFDF1	GNAS	M21142, 420–441	5′GTTTCAGGACCTGCTTCGC<u>A</u>GC3′
mutHGNASF1	GNAS	M21142, 420–441	5′GTTTCAGGACCTGCTTCGC<u>G</u>GC3′
HGNASR2	GNAS	M21142, 629–650	5′AGCACTGGATCCACTTGC<u>G</u>GCG3′
HBGPF1	osteocalcin	X51699, 143–167	5′GCAGCGAGGTAGTGAAGAGACCCAG3′
HBGPR1	osteocalcin	X51699, 282–306	5′GAAGCGCCGATAGGCCTCCTGAAAG3′
HBSPF1	osteopontin	X13694, 31–55	5′CTAGGCATCACCTGTGCCATACCAG3′
HBSPR1	osteopontin	X13694, 455–479	5′CCTCGGCCATCATATGTGTCTACTG3′
HALPF1	alkaline	X14174, 1603–1626	5′GCTGAGTGACACAGACAAGAAGCC3′
HALPR1	phosphatase	X14174, 2005–2028	5′GTGTGGGAAGTTGGCATCTGTCAC3′
HACTF1	β-actin	AB004047, 435–456	5′TGTA<u>T</u>GCCTCTGG<u>T</u>CGTACCAC3′
HACTR1	β-actin	AB004047, 1005–1026	5′ACAG<u>A</u>GTACTTGC<u>G</u>CTCAGGAG3′
HIL6F1	interleukin 6	M14584, 465–460	5′GAGTAGTGAGGAACAAGCCAGAGC3′
HIL6R1	interleukin 6	M14584, 729–752	5′TAAGTTCTGTGCCCAGTGGACACG3′

* Mismatched bases in the primer sequence are underlined.

PCR amplification of genomic DNA

Primers HGNASF1 and HGNASR1 (Table 2) were chosen from the guanine nucleotide-binding protein alpha subunit (*GNAS*) genomic sequence[27] and used to amplify the region carrying the Arg201 mutation. Five hundred nanograms of genomic DNA was amplified under 50 μl of oil in a 50 μl reaction that contained 1× Taq buffer (16.6 mM Tris-HCl, pH 8.8, 170 μg/ml bovine serum albumin, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.8 mM EDTA, 10 mM BME), 5% dimethylsulfoxide, 25 pmol of each primer, 75 nmol deoxy-NTPs, and 1.25 U Taq polymerase. The temperature profile consisted of a 2-minute melting step at 94°C, an amplification step of 30 s at 94°C, 30 s at 55°C, and 1 minute at 65°C for 25–30 cycles, and a final extension step of 6 minutes at 65°C. PCR products were size-separated on 4% (3:1 NuSieve/SeaKem, FMC Corp., Rockland, ME, U.S.A.) agarose gels and stained with ethidium bromide. Gel images were transferred to an Apple MacIntosh Quadra 800 via an Eagle Eye still video imaging system (Stratagene, La Jolla, CA, U.S.A.), and the relative band intensities analyzed with NCSA Gelreader version 2.07 software (National Center for Supercomputing Applications, 1991).

Restriction enzyme analysis

The His201 mutation, which creates a *Nla*III restriction enzyme site in the PCR amplified product,[5] was detected using primer pair HGNASF1/HGNASR1 as described above (Table 2). The wild-type PCR product is cut once by *Nla*III and yields 163 bp and 26 bp products, whereas the PCR product carrying the His201 mutation results in digested products of 94, 69, and 26 bp. A different forward primer (HGNASFDF1; Table 2) was designed to detect the Cys201 mutation. This primer results in the creation of a *Pvu*II site in the PCR product carrying the Cys201 mutation. The normal PCR product is not cut with *Pvu*II and yields a 146 bp fragment, whereas the mutant PCR product yields products of 125 bp and 21 bp. Ten microliters of the PCR reaction was added to 10 μl of dH_2O, and 0.5 μl of *Nla*III or *Pvu*II (New England Biolabs, Beverly, MA, U.S.A.) was added. The samples were incubated at 37°C for 1 h, and then half the sample was electrophoresed on a 4% agarose gel, followed by staining with ethidium bromide.

Subcloning and sequencing of PCR products

A forward primer (mutHGNASF1; Table 2) was used with the HGNASR2 reverse primer to create a restriction site in the normal PCR product of 231 bp. Neither the Cys201 mutant fragment nor the His201 mutant fragment is cut with *Eag*I; however, the normal PCR products yield fragments of 19 bp and 212 bp. PCR products were cut with *Eag*I (New England Biolabs) according to the manufacturer's instructions. Samples were electrophoresed on a 4% agarose gel, followed by staining with ethidium bromide. The undigested product was picked with a sterile plastic toothpick and further amplified by stirring the toothpick in a fresh 50 μl of PCR reaction for 5 s and then PCR amplified as described above. Uncut fragments were ligated into the pCR cloning vector from the TA cloning kit (Invitrogen, San Diego, CA, U.S.A.), according to the manufacturer's instructions. Ligated plasmids were transformed into Epicurian coli XL-1 blue competent cells (Stratagene, LaJolla, CA, U.S.A.) by standard methods.[28] Sequencing of the cloned inserts was performed by using the Sequenase kit (version 2.0; U.S. Biochemicals, Cleveland, OH, U.S.A.) according to the protocol for double-stranded DNA.

RNA isolation and reverse transcription-PCR analysis

Total RNA was isolated from control and treated cells that were grown in 100-mm tissue culture dishes (at conflu-

ency, ~10^6 cells) by using the RNAgents kit from Promega (Madison, WI, U.S.A.). The preparations were quantitated and their purity determined by standard spectrophotometric methods. For reverse transcription (RT)-PCR analysis, 1 μg of total RNA was brought up to 10 μl in diethylpyrocarbonate-treated water. The sample was heated to 75°C for 3 minutes, placed on ice, and cDNA synthesis was performed by RT for 15 minutes at 42°C in a 20 μl reaction containing 1× PCR buffer II (Perkin-Elmer, Corp., Norwalk, CT, U.S.A.), 5 mM $MgCl_2$, 1.25 mM each dNTP, 1 U/μl RNAsin (Promega), 12.5 μg/μl oligo $(dT)_{15}$, and 2.5 U/μl AMV RT (Promega). Subsequent amplification of the cDNA sequence was performed with either 1, 2, or 10 μl of the reverse transcription reaction in 1× Taq buffer, 5% dimethylsulfoxide, 25 pmol each primer (Table 2), except for the β-actin primers which were added at 5 pmol per reaction, and 1.25 U of Taq polymerase in a 50-μl reaction volume. For assessment of the relative mRNA levels of IL-6 to β-actin, a multiplex RT-PCR approach was used as described by Dukas et al.[29] The PCR reaction was done as described above with 25 pmol/μl each IL-6 primer and 5 pmol/μl each β-actin primer. The temperature profile for the PCR reactions consisted of a 2 minute melting step at 94°C, followed by 30 s at 94°C, 30 s at 55°C, and 1 minute at 65°C for the number of cycles indicated in the figures, and a final extension step of 6 minutes at 65°C. RT-PCR products were separated by size on a 4% agarose gel and stained with ethidium bromide. Images were transferred to an Apple MacIntosh Quadra 800 via an Eagle Eye still video imaging system, and the relative band intensities analyzed with National Institutes of Health Image software (Bethesda, MD, U.S.A.).

IL-6 production by the cultured stromal cells

Bone marrow stromal cells from either normal or FD patients were plated from frozen stocks at 5×10^3 cells/cm^2 in 100-mm tissue culture dishes in α-MEM supplemented with 15% FBS and penicillin-streptomycin. At confluence, 50 μg/ml L-ascorbate, 10 mM β-glycerophosphate, and either 10 nM dexamethasone or 100 nM methylprednisolone was added to the medium, and the medium was changed every other day. The medium from each cell feeding was collected and stored frozen at –70°C until analysis. Samples were thawed at 4°C overnight and then centrifuged at 13,000 rpm for 5 minutes. The supernatant (100 μl) was assayed for IL-6 levels by use of an IL-6 ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's recommendations.

RESULTS

Culturing of bone marrow stromal cells from normal and FD patients

Utilizing tissue culture techniques that have been developed for rat bone marrow stromal cells,[15,16] we successfully cultured stromal cells from 11 FD patients and three normal controls. In contrast to a report by Shenker et al.,[10] we found that the cultured FD cells grew slower than cells cultured from unaffected controls. Control cells from two patients grew with an average doubling time of 14 h, whereas the cells from four FD patients had an average doubling time of 22 h. The difference in population doublings was not related to the age of the patients because the two control cell lines used were from patients 6.8 years and 12.5 years of age, whereas cells from four of the FD patients analyzed were 6.3 (Case 2), 7.0 (Case 3), 7.5 (Case 4), and 11.1 (Case 6) years old at the time of surgery (Table 1). Cells cultured from the oldest FD patient (age 19.1; Case 1) had the slowest doubling time of 39 h.

Detection of the $G_s\alpha$ mutations in cultured FD cells

The $G_s\alpha$ mutation has been shown to be present in varying amounts in affected tissues of FD patients; however, microdissection of some of the affected tissues has shown that the mutant gene is more abundant in abnormally proliferating regions of the tissue than in the normal regions.[9] To ensure that we could detect even a low frequency of the mutation in cultured cells from the FD patients, we developed a sensitive PCR-based detection assay. The His201 mutation creates a new *Nla*III restriction site, which has been utilized by Malchoff et al.[5] to detect this mutation in affected bone tissue from a patient with atypical McCune–Albright syndrome. Since the Cys201 mutation does not create or abolish a restriction site, we engineered a site by modifying a PCR primer with a single base pair change near the 3′ end of the primer (HGNASFDF1; Table 2). By using this modified forward primer to amplify DNA, a *Pvu*II restriction site was obtained from the DNA of cells with the Cys201 mutation.

DNA was isolated from the cultured cells of both normal and FD patients, and the region encompassing the $G_s\alpha$ mutation was amplified by PCR as described in the Materials and Methods. The amplified DNA was then subjected to restriction enzyme digestion with either *Pvu*II or *Nla*III, and the digested products separated by electrophoresis, followed by ethidium bromide staining. The ratio of cut to uncut product gives an estimate of the proportion of cells that contain the $G_s\alpha$ mutation (e.g., if 100% of the cells contained the mutation, a ratio of 1:1 for the mutant and wild-type PCR product would be expected). However, this type of analysis most likely underestimates the frequency of the mutation, due to an inability of the restriction enzymes to cut heteroduplex PCR products consisting of both normal and mutant DNA strands. Also, estimation of the Cys201 mutation by *Pvu*II cleavage may be underestimated because of inefficiency of cutting close to the end of the DNA fragments. Indeed, we have performed a similar type of restriction analysis on the amplified DNA from patients known to be heterozygous for a point mutation in a chloride channel gene (unpublished results) and shown that the technique provides an estimate of the mutation frequency to be 0.60 ± 0.05 ($n = 7$), rather than the expected frequency of 1.0. Nevertheless, this analysis can provide a relative comparison of the percentage of cultured stromal cells carrying the $G_s\alpha$ mutation from the three types of FD. Figure 2A shows that two patients with McCune–Albright syndrome (Cases 2 and 6) carry the Cys201 mutation in most of their cultured cells, whereas a male with polyostotic

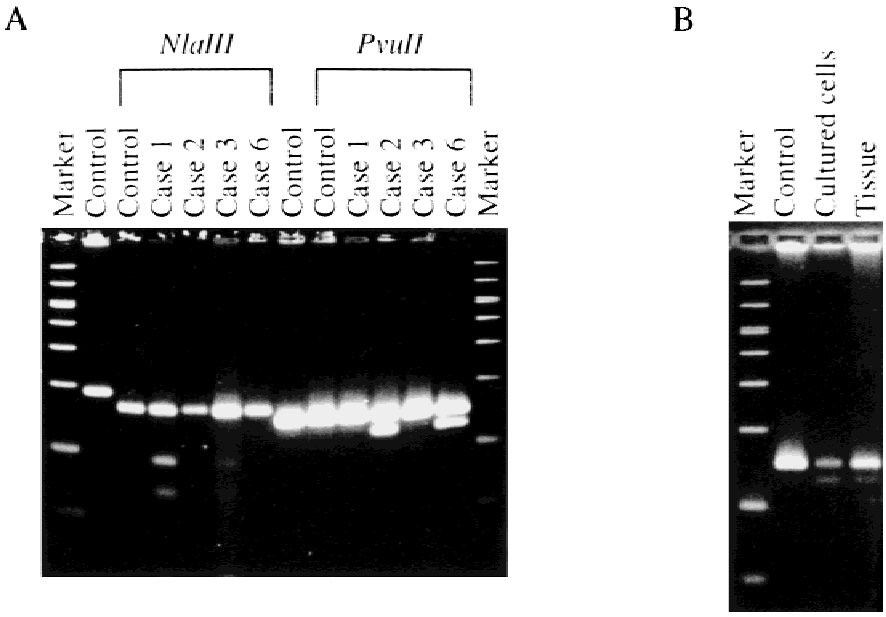

FIG. 2. Nonisotopic detection of the $G_s\alpha$ mutation in FD patients. (A) DNA prepared from cultured cells of either FD patients or normal controls was amplified using primers designed to detect either the His201 mutation after *Nla*III digestion or the Cys201 mutation after *Pvu*II digestion, as described in Materials and Methods. Bands of 94 bp and 69 bp after digestion of DNA from Case 1 and Case 3 with *Nla*III indicate that they carry the His201 mutation. A band of 125 bp after digestion of DNA from Case 2 and Case 6 with *Pvu*II indicates that they carry the Cys201 mutation. (B) DNA prepared from either cultured cells or tissue of Case 2 was amplified using primers designed to detect the Cys201 mutation after digestion with *Pvu*II. Markers: 1000, 700, 525, 500, 400, 300, 200, 100, and 50 bp.

FD (Case 1) carries the His201 mutation in the majority of his cultured cells. Interestingly, a low frequency of the His201 mutation was found in cultured stromal cells from two male patients with monostotic FD (Cases 3 and 4). Similar analyses of the mutation in the other FD cell lines (Table 1) show that all patients affected with either McCune–Albright syndrome or polyostotic FD had a high frequency of the $G_s\alpha$ mutation, with the exception of a male patient with McCune–Albright syndrome (Case 9) who did not show either the His201 or Cys201 mutation. All of the patients with monostotic FD showed a lower frequency of the mutation than those with polyostotic FD. Neither $G_s\alpha$ mutation was found in the cultured stromal cells from three normal patients. Thus, in this limited study, a correlation between frequency of the mutation and progressive or recurrent disease was seen.

The presence of the $G_s\alpha$ mutation was verified by subcloning and sequencing of the region containing the mutation in four of the patients. Since the frequency of the mutation was low in some patients, we enriched for the PCR product carrying the mutation before subcloning. Primer mutGNASF1, which has a single base change near its 3′ end, creates an *Eag*I site when used to amplify the normal sequence. When patient DNA was amplified with the mutHGNASF1/HGNASR2 primer pair and the PCR product was digested with *Eag*I, the uncut fragment would presumably carry either the Cys201 or His201 mutation. The uncut mutant fragment was further amplified, subcloned, and sequenced. In addition, since the *Eag*I enzyme did not cut to completion, we were also able to subclone and sequence the normal fragment. Figure 3 shows the presence of the Cys201 mutation (C→T) in Case 6 and the His201 mutation (G→A) in Case 3. The His201 mutation in Case 1 and the Cys201 mutation in Case 2 were also verified by sequence analysis (data not shown).

Detection of the $G_s\alpha$ mutation in FD tissue

DNA was isolated directly from the affected stromal tissue of a McCune–Albright syndrome patient (Case 2), who was shown to have the Cys201 mutation in her cultured cells. The mutation was detected as described above, and the results shown in Fig. 2B. The average frequency of cells carrying the mutation in two independent determinations of DNA isolated from the affected tissue was 23%, which was about 72% lower than the estimated frequency in the cultured cells. The lower frequency is most likely due to the presence of hematopoietic cells in the fresh stroma that do not carry the $G_s\alpha$ mutation; these hematopoietic cells are lost as the adherent cells from the affected stroma are maintained in culture.[15–18] In support of this hypothesis, several studies[5,9] have failed to detect the $G_s\alpha$ mutation in peripheral lymphocytes from even severely affected FD patients.

Glucocorticoid-induced mineralization of cultured FD cells

Glucocorticoids, such as hydrocortisone and dexamethasone, have been shown to be essential in vitro for conver-

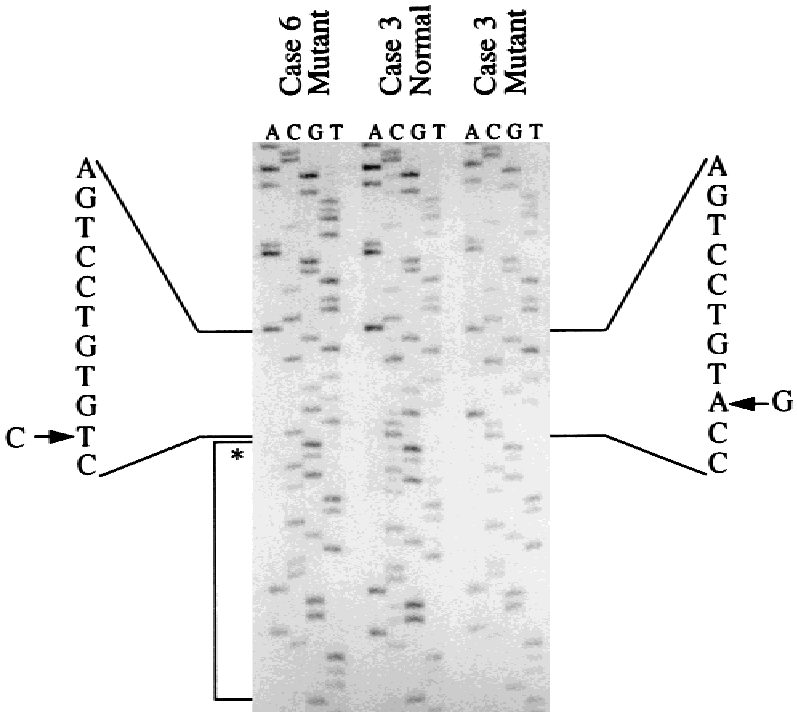

FIG. 3. Detection of the $G_s\alpha$ mutation in FD patients by sequence analysis. DNA prepared from cultured cells of FD patients was amplified with primers designed so that only the normal allele is digested with *Eag*I. After partial digestion with *Eag*I and further amplification, both the normal and mutant alleles were subcloned and sequenced as described in Materials and Methods. The C→T transition of Case 6 and the G→A transition of Case 3 are indicated. The bracket indicates sequence of the primer used to amplify the DNA. The asterisk indicates the mismatched G in the primer, which creates the *Eag*I restriction site in the normal allele.

sion of cultured stromal cells to osteoblasts, which mineralize in tissue culture.[15–17,19] Figure 4 shows that both dexamethasone and methylprednisolone induced mineralization of cultured stromal cells from normal and FD patients. Dose response curves, as determined by alizarin red and von Kossa staining, revealed that dexamethasone was optimal at 10 nM, whereas methylprednisolone was optimal at 100 nM (data not shown). Figure 5 shows that both the normal and FD cells expressed osteogenic markers, such as osteocalcin, osteopontin, creatine kinase B,[30] and bone/liver/kidney ALP mRNAs. β-actin mRNA was amplified in parallel to ensure that differences in amplification product were not the result of differences in the amount of input RNA. ALP mRNA levels were estimated to be at least 5- to 10-fold more abundant in the FD cells as compared with the normal cells, when the number of amplification cycles, the amount of input cDNA, and the relative intensities of the bands were considered. This agrees with the results of two other studies,[2,3] in which stromal cells in the fibrotic areas of FD were shown to express ALP. IL-6 mRNA was also expressed in both the normal and FD cell lines, with levels being 3.7-fold higher in the FD cells obtained from the McCune–Albright syndrome patient (Case 8) as compared with normal cells. The levels of IL-6 mRNA in Case 10 were similar to those of the normal cells. It is relevant to note that the clinical severity of Case 10 was mild as compared with Case 8 (Table 1), and the frequency of the $G_s\alpha$ mutation was lower in Case 10 (39%) than in Case 8 (63%). Both dexamethasone and methylprednisolone dramatically decreased IL-6 mRNA levels in the control and FD cell lines while in general, increasing ALP, creatine kinase B, and osteocalcin mRNA levels. IL-6 mRNA levels in dexamethasone-treated control, Case 8, and Case 10 cells were 36, 1, and 26%, respectively, of untreated cells. In general, glucocorticoids decreased osteopontin mRNA levels, while having no effect on β-actin mRNA. The relative level of IL-6 to β-actin mRNA in the cultured cells was also determined by multiplex RT-PCR for 25 cycles, which was in the linear phase of the PCR assay (data not shown), as described in the Materials and Methods. The results (Fig. 6) show that glucocorticoids decrease IL-6 mRNA in relation to β-actin by an average of 46% in control and 74% in FD (Case 8) cells.

Effect of glucocorticoids on IL-6 secretion

Both normal and FD cells were exposed to either 10 nM dexamethasone or 100 nM methylprednisolone for 6 days. The culture medium was collected every 2 days and analyzed for IL-6 by ELISA as described in the Materials and Methods. As reported by Yamamoto et al.,[24] cells cultured from the McCune–Albright syndrome patient (Case 8) secreted significantly higher levels of IL-6 into the culture medium as compared with normal cells (Table 3). Interestingly, IL-6 levels in medium collected from the polyostotic FD patient (Case 10) were only slightly higher than those from normal cells. The data in Table 3 also show that glucocorticoids decrease IL-6 levels by about 2-fold in cell culture supernatants from both the normal and FD cells. The relative levels of IL-6 secretion paralleled the relative differences in IL-6 mRNA expression in the normal and FD cell lines (Fig. 6 and Table 3).

DISCUSSION

In this study, we have demonstrated the feasibility of culturing osteogenic cells from the affected lesions of FD

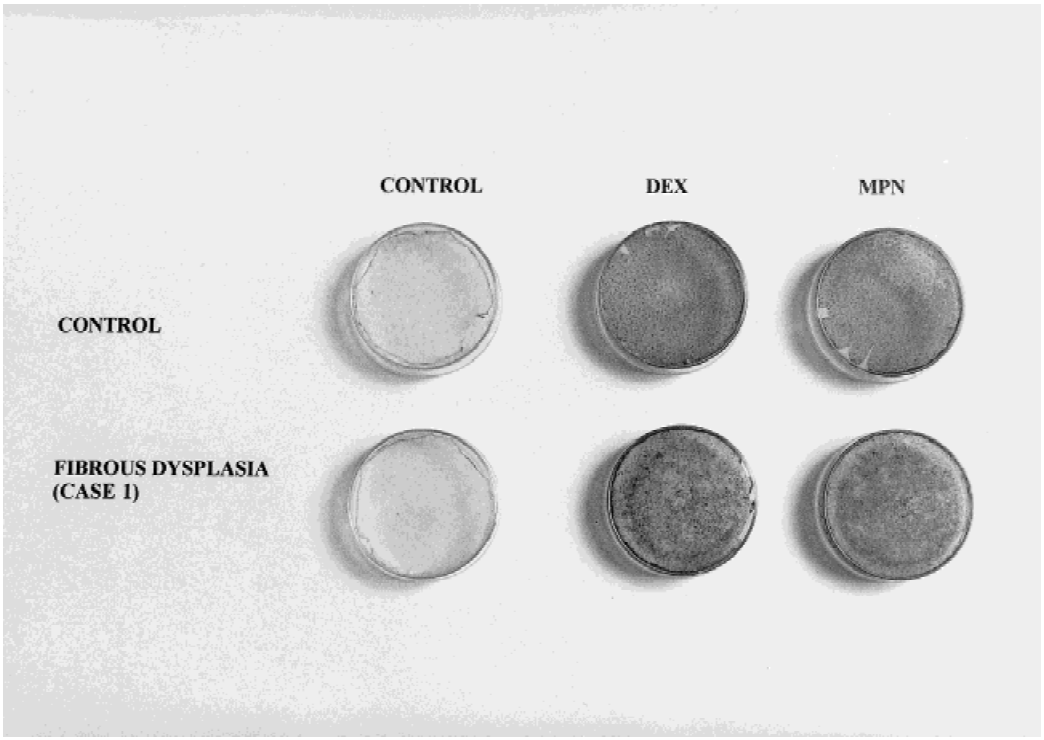

FIG. 4. Glucocorticoid-induced mineralization of cultured normal and FD (Case 1) cells. Either 10 nM dexamethasone or 100 nM methylprednisolone was added to cells on day 1. At day 21 in culture, cells were stained with 2% alizarin red as described in the Materials and Methods.

patients. A nonradioactive PCR-based assay was used to demonstrate the presence of the $G_s\alpha$ mutation in the cultured FD cells. We show here that osteogenic cells cultured from the affected marrow stroma of five patients with McCune–Albright syndrome and two patients with polyostotic FD showed a high frequency of the $G_s\alpha$ mutation. In contrast, cells from the three patients with monostotic FD showed a low frequency of the mutation. These results agree with the study of Candeliere et al.,[31] which showed that the frequency of the $G_s\alpha$ mutation is extremely low in peripheral blood lymphocytes from monostotic as compared with polyostotic FD. In this study, the patients with monostotic FD showed no recurrence of the dysplasia within a 2-year period, whereas the majority of the McCune–Albright syndrome and polyostotic FD patients required repeated corrective surgery for either recurrent disease at previously affected sites, or additional surgery at other sites of involvement. Thus, in this limited study, a correlation between frequency of the mutation and progressive or recurrent disease was seen. It will be interesting to culture cells from additional FD patients to see if this relationship holds true and can serve as an useful prognostic indicator for the clinical severity of the disease.

Bianco et al.[32] have recently developed an animal model of FD by transplanting a mixture of normal and $G_s\alpha$-mutated human osteogenic precursor cells into immunocompromised mice. Interestingly, transplantation of clonal populations of mutant cells resulted in loss of the transplanted cells and no ossicle formation, in contrast to the normal ossicle formation seen after transplantation of clonal populations of normal cells. Thus, the development of FD appears to be dependent on the presence of both normal and mutant cells. In agreement with these data, we found that the frequency of the $G_s\alpha$ mutation in cells cultured from FD lesions was never 100% (Table 1). However, we did observe an increased proportion of mutant to normal cells from the more severe lesions, suggesting that the relative ratio of normal to mutant cells influences the severity of the lesion. This hypothesis can be tested in the animal model of FD.[32]

Neither the Cys201 nor His201 mutation was detected in one of the McCune–Albright syndrome patients (Case 9). Furthermore, a Ser201 mutation, which was found in one patient with panostotic FD,[31] was also not detected in this patient (data not shown). However, it is possible that an as yet unidentified mutation is the cause of FD in Case 9. In support of this possibility is a recent study by Gessl et al.,[33] which showed a functionally normal $G_s\alpha$ protein in an atypical McCune–Albright syndrome patient with polyostotic FD, acromegaly, and pituitary adenoma. DNA analysis of affected tissue from this patient also failed to detect either of the two known activating mutations in the *GNAS* gene. These authors propose that an activating mutation, which acts downstream from $G_s\alpha$ in the regulatory cascade, could be the cause of FD in this patient and in a subset of other FD patients.

Similar to stromal cells cultured from the bone marrow of other mammals, the cultured stromal cells from FD patients

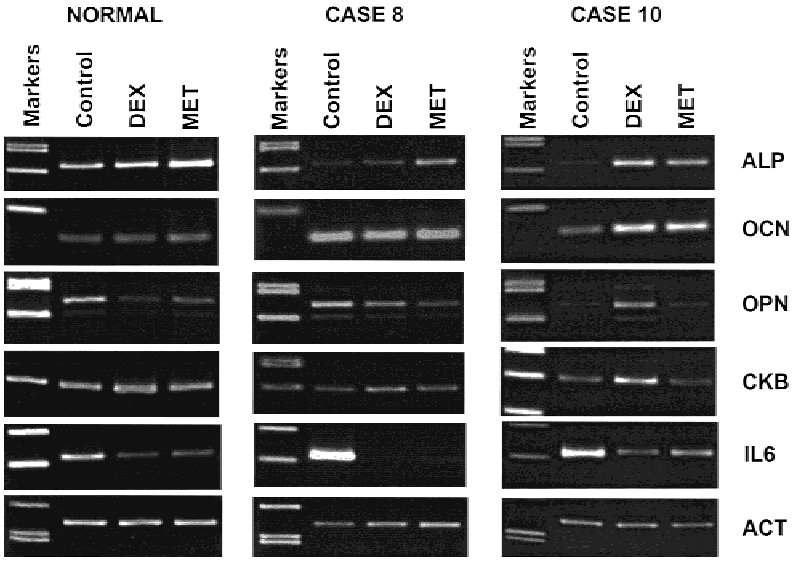

FIG. 5. Effect of glucocorticoids on mRNA expression in control and FD cells. Cultured stromal cells from either normal or FD (Cases 8 and 10) patients were seeded into 100 mm tissue culture dishes. Either 10 nM dexamethasone or 100 nM methylprednisolone was added on day 1 forward as described in the Materials and Methods, and cells were fed every other day. On day 14 in culture, total RNA was isolated and analyzed for expression of osteocalcin (OCN), osteopontin (OPN), alkaline phosphatase (ALP), creatine kinase B (CKB), interleukin-6 (IL-6), and β-actin (ACT) mRNA expression by RT-PCR as described in the Materials and Methods using 10 μl of resulting cDNA for 25 cycles (Cases 8 and 10) or 30 cycles (Control) for ALP; 10 μl for 25 cycles (Control and Case 10) or 27 cycles (Case 8) for OCN; 10 μl for 25 cycles (Control), 27 cycles (Case 10) or 30 cycles (Case 8) for OPN; 1 μl for 25 cycles for CKB; 2 μl for 25 cycles (Control and Case 8) or 28 cycles (Case 10) for IL-6; and 1 μl for 25 cycles (Control and Case 10) or 27 cycles (Case 8) with primers diluted 1:5 for ACT. RT-PCR analysis of total RNA from (A) Control patient; (B) FD patient, Case 8; (C) FD patient, Case 10. Lane 1, no treatment; lane 2, 10 nM dexamethasone; lane 3, 100 nM methylprednisolone. Sizes of the RT-PCR products are 426 bp for ALP, 164 bp for OCN, 449, 407, 368 bp for OPN a (GenBank accession no. D28759), b (GenBank accession no. D28760), c (GenBank accession no. D28761) transcripts, respectively, 404 bp for CKB, 316 bp for IL-6, and 592 bp for β-actin.

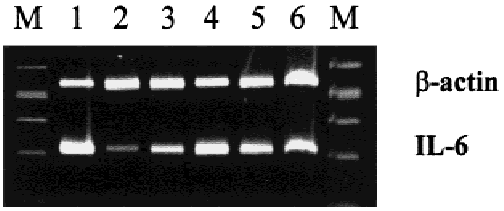

FIG. 6. Effect of glucocorticoids on IL-6 mRNA levels in relation to β-actin mRNA in normal and FD cells. Cell culture and RNA isolation were as described in the legend to Fig. 5. The relative levels of IL-6 mRNA to β-actin mRNA were determined by multiplex RT-PCR for 25 cycles as described in the Materials and Methods. Lanes 1–3, FD cells (Case 8), no treatment, 10 nM dexamethasone, 100 nM methylprednisolone, respectively. Lanes 4–6, normal cells, no treatment, 10 nM dexamethasone, 100 nM methylprednisolone, respectively. Markers (M): 700, 525, 500, 400, and 300 bp. Sizes of the RT-PCR products are 316 bp for IL-6 and 592 bp for β-actin.

TABLE 3. EFFECT OF GLUCOCORTICOIDS ON IL-6 SYNTHESIS IN CULTURED NORMAL AND FD CELLS*

Cell line	*Treatment*	*IL-6 levels (ng/ml)*
Normal	none	1467 ± 62
	dexamethasone	849 ± 68
	methylprednisolone	585 ± 23
Case 8	none	6038 ± 346
	dexamethasone	2670 ± 1225
	methylprednisolone	2472 ± 18
Case 10	none	1638 ± 64
	dexamethasone	804 ± 170
	methylprednisolone	704 ± 20

* Culture media was obtained from confluent cells exposed to either 10 nM dexamethasone or 100 nM methylprednisolone for 6 days. IL-6 levels were determined by ELISA as described in the Methods. Values are the mean ± SD of duplicate determinations and show secreted IL-6 levels over 48 h.

required dexamethasone addition for mineralization in tissue culture. It is interesting to note that the cultured FD cells mineralized in the presence of dexamethasone in vitro in a fashion similar to normal human bone marrow cells. In vivo, however, the FD stromal cells fail to produce clinically effective mineralization. It has been postulated that the $G_s\alpha$ mutation results in enhanced proliferation and abnormal differentiation of a preosteoblastic stem cell.[3,10] However, we have shown that the cultured FD cells actually proliferate slower than normal cells. Furthermore, we have observed a decrease in the frequency of the $G_s\alpha$ mutation

as cells are passaged in culture (unpublished data), which would be expected if the mutant cells actually grow slower than the normal cells. The most likely explanation for our findings is that FD cells senesce in culture more rapidly than normal cells. Earlier senescence is probably caused by the increased proliferation of the FD cells in vivo. This would be analogous to the situation in Duchenne muscular dystrophy where satellite cells from dystrophic muscle senesce earlier in culture than normal cells, because of the increased proliferation of satellite cells in regenerating dystrophic muscle.[34]

Glucocorticoids, such as dexamethasone and methylprednisolone, enhanced osteoblastic differentiation of the cultured FD cells, as evidenced by increased expression of bone-specific proteins and induction of mineralization in the treated cells (Figs. 4 and 5). These results indicate that a similar type of preosteoblastic stem cell that has been described in the rat and more recently, in man[18,35] and pig[17] appears to be present in the stromal cell cultures that we have established from individuals with FD. Bianco et al.[32] have also confirmed the presence of marrow stromal cells in the fibrotic lesions of McCune–Albright syndrome patients.

The enhancement of osteogenic differentiation in both the normal and FD cells can be explained by the ability of glucocorticoids to decrease IL-6 mRNA levels, since IL-6 has been shown to inhibit bone formation in vitro.[20] Increased IL-6 expression in FD is most likely due to increased c-*fos* expression,[36] which in turn is caused by the constitutive expression of cAMP. Increased IL-6 expression also causes increased osteoclast activity.[21–23] Treatment of human bone marrow stromal cells with glucocorticoids results in production of a protein that degrades IL-8 mRNA, presumably by binding to an AUUUA sequence in the 3′-untranslated region of the mRNA.[37] This same binding element is present in the 3′-untranslated region of IL-6 mRNA.[38] We demonstrate here (Figs. 5 and 6 and Table 3) that glucocorticoids decrease both IL-6 mRNA levels as well as secreted IL-6 protein in both the normal and FD cells. This cascade of events explains why glucocorticoid treatment could partially overcome both the abnormal osteoblast differentiation and increased osteoclastic activity caused by an activating G protein mutation in FD.

In summary, we have demonstrated the feasibility of culturing stromal cells, which carry the $G_s\alpha$ mutation, from FD patients. Since these individuals often require surgical intervention for treatment of their dysplasia, the removed tissue provides a convenient source of DNA for verification of the $G_s\alpha$ mutation, because the mutation has been rarely detected in peripheral blood lymphocytes. An assessment of the severity of the disease may also be gleaned from determination of the frequency of the $G_s\alpha$ mutation in cultured cells. Finally, the cultured cells will provide a useful system in which to test putative therapeutic agents for their ability to modify the FD phenotype, as demonstrated here for glucocorticoid-mediated enhancement of osteoblast differentiation in FD cells. Putative therapeutic agents may then be tested in a recently developed animal model of FD.[32]

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